(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 527 728 B1

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:
09.07.1997 Bulletin 1997/28

(21) Application number: **90908867.6**

(22) Date of filing: **13.04.1990**

(51) Int Cl.[6]: **C12P 19/34**, C12Q 1/68, C12N 9/12, C07H 15/12, C07H 17/00

(86) International application number:
PCT/US90/01631

(87) International publication number:
WO 91/16446 (31.10.1991 Gazette 1991/25)

(54) **(IN VITRO) DNA SYNTHESIS REACTIONS USING MODIFIED PHI 29 DNA POLYMERASE AND A DNA FRAGMENT ENCODING SAID POLYMERASE**

IN VITRO-DNS-SYNTHESEREAKTIONEN UNTER VERWENDUNG VON GEÄNDERTEM PHI-29-DNS-POLYMERASE UND FÜR BESAGTE POLYMERASE KODIERENDES DNS-BRUCHSTÜCK

REACTIONS DE SYNTHESE D'ADN (IN VITRO) UTILISANT LA POLYMERASE DE PHI 29 ADN MODIFIEE, ET UN FRAGMENT D'ADN CODANT LADITE POLYMERASE

(84) Designated Contracting States:
AT BE CH DE DK ES FR GB IT LI LU NL SE

(43) Date of publication of application:
24.02.1993 Bulletin 1993/08

(73) Proprietors:
- **CONSEJO SUPERIOR DE INVESTIGACIONES CIENTIFICAS**
  E-28006 Madrid (ES)
- **UNITED STATES BIOCHEMICAL CORPORATION**
  Cleveland, OH 44128 (US)

(72) Inventors:
- **BLANCO, Luis**
  Avenida Prado de Los Rosales, 48
  E-Madrid (ES)
- **BERNAD, Antonio Calle Rocafort, 135-5**
  E-28021 Madrid (ES)
- **SALAS, Margarita**
  E-28003 Madrid (ES)

(74) Representative: **Sexton, Jane Helen et al**
J.A. KEMP & CO.
14 South Square
Gray's Inn
London WC1R 5LX (GB)

(56) References cited:
EP-A- 0 265 293
WO-A-90/10064
WO-A-90/12111
WO-A-91/03573
US-A- 4 683 202
US-A- 5 001 050

- **CELL vol. 59 , 6 October 1989 , NEW YORK US pages 219 - 228 BERNAD ET AL. 'A conserved 3'-5' exonuclease active site in DNA polymerases'**
- **Nucleic Acid Res.(1985) 13(5) 1239-1248**
- **BLANCO et al., EMBO Workshop, pg 63, July 1988, "Processive replication by the bacteriophage PHI-29 DNA polymerase".**
- **BLANCO et al., Gene, Vol. 29, pages 33-40, (see Abstract), "Cloning and expression of gene 2, required for the protein-primed initiation of the Bacillus subtilis PHI-29 DNA replication", (01 March 1984).**
- **BLANCO et al., Proc. Natl. Acad. Sci., Vol. 81, pg. 5325-5329, "Characterization and purification of a PHI-29 encoded DNA polymerase required for the initiation of replication", (see Abstract) (1984).**
- **SANGER et al., Proc. Natl. Acad. Sci., Vol. 74, No. 12, pp. 5463-5467, "DNA sequencing with chainterminating inhibitors", (see Abstract) (December 1977).**
- **GUTIERREZ et al., Nucleic Acids Research, Vol. 16, No. 13, 10 June 1988, pp. 5895-5913, "Characterization of the origins of replication of bacteriophage PHI-29 DNA", (see Abstract).**

Printed by Jouve, 75001 PARIS (FR)

(Cont. next page)

- GUTIERREZ et al., Gene, Vol. 43, pp. 1-11, 27 January 1986, "Cloning and template activity of the origins of replication of PHI-29 DNA", (see Abstract).
- BLANCO et al., Proc. Natl. Acad. Sci., Vol. 82, pp. 6404-6408, October 1985, "Replication of PHI-29 DNA with purified terminal proetin and DNA polymerase: Synthesis of full-length PHI-29 DNA", (see Abstract).
- PRIETO et al., Proc. Natl. Acad. Sci., Vol. 181, pp. 1639-1643, (March 1984), "Purification in a functional form of the terminal protein Bacillus subtilis PHI-29".
- YASHIKAWA et al., Gene, Vol. 37, pp. 125-130 (01 April 1985), "Nucleotide sequence analysis of DNA replication origins of the small Bacillus bacteriophages: evolutionary relationships".

Description

This invention relates to use of modified ϕ29-type DNA polymerases, in particular ϕ29-type DNA polymerases modified so as to have little or no exonuclease activity.

By ϕ29-type DNA polymerase is meant any DNA polymerase isolated from cells infected with a ϕ29-type phage which employs a terminal protein for the initiation of replication of DNA. These phages are generally described by Salas, 1 in The Bacteriophages 169, 1988. These phages are closely related in the structure of their DNA polymerases, some differing by as few as 6 amino acid changes with 5 of those amino acids being replaced by similar amino acids. These phages have a short inverted terminal repeat sequence of length between about 6 and 300 nucleotides. These polymerases have a highly active 3'-5' exonuclease activity, but no 5'-3' exonuclease activity. Surprisingly, although they are related to the T4 family of DNA polymerases, they are able to adequately recognize chain terminating agents such as dideoxynucleosides and therefore are useful for DNA sequencing. This ability is even more surprising since the exonuclease is known to recognize both deoxy and dideoxy ADP. Blanco *et al,* 13 Nuc. Acid. Res. 1239, 1246, 1985.

By way of example, ϕ29-type DNA polymerases suitable for modification to provide a mutant ϕ29-type DNA polymerase for the purpose of the methods disclosed herein include ϕ29, Cp-1, PRD1, ϕ15, ϕ21, PZE, PZA, Nf, M2Y, B103, SF5, GA-1, Cp-5, Cp-7, PR4, PR5, PR722 and L17. A mutant ϕ29-type DNA polymerase for the purpose of the present invention is a modified polymerase having less than 10% of the exonuclease activity of the naturally-occurring polymerase. Preferably, the modified polymerase has less than 1 %, and even more preferably has substantially no exonuclease activity compared with the corresponding naturally-occurring polymerase.

By corresponding is meant that the modified polymerase is derived from a naturally-occurring polymerase, generally by *in vitro* mutagenesis of the DNA sequence encoding the latter polymerase, and the latter is the corresponding polymerase.

For this purpose, a DNA fragment encoding an appropriate natural polymerase may be modified to substantially eliminate the naturally-occurring exonuclease activity of that expression product. DNA sequences encoding a ϕ29 DNA polymerase in which the amino acid moiety at position 12, 14 or 16 of the polymerase is replaced by an alanine moiety may be employed.

In one aspect, the present invention thus provides a method for amplification of a DNA sequence including the steps of annealing a first primer and a second primer to opposite strands of a double-stranded DNA sequence and incubating the annealed mixture with a DNA polymerase, wherein the DNA polymerase employed is a modified ϕ29-type DNA polymerase exhibiting less than 10% of the exonuclease activity of the corresponding naturally-occurring polymerase.

In preferred embodiments, the first and second primers have their 3' ends directed towards each other after annealing; the method further includes, after the incubation step, denaturing the resulting DNA, annealing the first and second primers to the denatured DNA and incubating the last annealed mixture with the polymerase; the cycle of denaturing, annealing and incubating is repeated from 10-40 times.

In a further aspect, the invention provides a method for production of DNA molecules of greater than 10 kilobases in length. The method includes providing a template DNA molecule; annealing a primer with the template molecule; and incubating the annealed primer and template molecules in the presence of a modified ϕ29-type DNA polymerase as hereinbefore described and a mixture of four different deoxynucleoside triphosphates.

The high processivity retained by a modified ϕ29-type DNA polymerase as hereinbefore described means that an amplification method of the present invention is suitable for amplifying very large fragments of DNA (in excess of 10 kilobases in length). Such an amplification method may be a polymerase chain reaction (PCR)-type procedure. These long lengths of DNA are of use in forensic work, when small samples of DNA are available, and for restriction fragment length polymorphism analysis.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

The drawing will first briefly be described.

Drawing

The Figure is a represention of the amino acid sequence of various DNA polymerases showing sites of homology between the polymerases. ExoI, ExoII and ExoIII refer to the three regions of amino acid homology found among the different DNA polymerases compared in the Figure. Stars indicate the E. coli DNA polymerase I residues involved in either metal binding, or exonucleolytic catalysis. Asterisks indicate the E. coli DNA polymerase I residues involved in single-stranded DNA binding. Boxes shown by lines or arrows, and lettered or numbered, are α-helix and β-sheet regions respectively of DNA polymerase I.

DNA Polymerase

A modified DNA polymerase for use in a method of the invention is processive and has little or no associated exonuclease activity (less than 10% of the exonuclease activity exhibited by the corresponding native

polymerase). These polymerases also have a strand-displacement activity.

By processive is meant that the DNA polymerase is able to continuously incorporate nucleotides using the same primer template, without dissociating from either or both the primer or the template molecules, under conditions normally used for DNA sequencing extension reactions, or other primer extension reactions. Generally, a polymerase for use in a method of the present invention will remain bound to the extended primer or template for at least 1-2 kilobases, generally at least 5kb-10kb, under suitable environmental conditions.

The ability of the polymerases to produce strand-displacement is advantageous because, in combination with high processivity, it allows synthesis of long DNA molecules of at least 70kb, or even greater. Strand displacement activity is measured by any standard technique, for example, a polymerase may be incubated in a mixture with a single-stranded circular DNA molecule (e.g., M13) and a primer. If DNA molecules of length greater than the original circular molecule are synthesized, then the polymerase is able to displace DNA strands of a double-stranded molecule and continue to synthesize DNA--thus, it has a strand displacement activity. Such activity is generally present in a single protein molecule, e.g., p2 of ϕ29, and does not require energy in the form of ATP or its equivalent, utilizing only the standard deoxynucleoside triphosphates required to synthesize DNA. This activity is also observed when DNA synthesis is initiated by a terminal protein, e.g., p3 of ϕ29.

It is preferred that the level of exonuclease activity be reduced to a level which is less than 1%, preferably less than 0.1% of the activity normally associated with DNA polymerases isolated from cells infected with naturally-occurring bacteriophage. Modification of a DNA polymerase for the purpose of the present invention may be performed by genetic or chemical means.

The examples below illustrate the invention with reference to ϕ29 DNA polymerase. The examples are not meant to be limiting to the invention. Those skilled in the art will recognize that any of the above enumerated DNA polymerases can be similarly used in the manner described below.

ϕ29 DNA Polymerase

Bacteriophage ϕ29 is a linear double-stranded DNA molecule having a protein of 31 kD covalently linked at the 5' end. This terminal protein, termed p3, is the product of viral gene 3, and is linked to the DNA by a phosphoester-bond between the OH group of a serine residue and 5'-dAMP. ϕ29 replication is initiated at either DNA end by a protein priming mechanism in which a free molecule of the terminal protein p3 reacts with dATP to form a protein-p3-dAMP covalent complex that provides the 3' OH group needed for elongation. The initiation reaction requires, in addition to the gene 3 product and the ϕ29 DNA-protein p3 template, the product of the viral gene 2 (p2), which is the DNA polymerase. Protein p2 produced from gene 2 has a molecular weight of 66.5 kD. Associated with protein p2 is a 3'-5' exonuclease activity active on single stranded and to some extent on double stranded DNA. Protein p2 may be purified by standard procedure from E. coli cells harboring a gene 2 containing recombinant plasmid, as described by Blanco et al., 29 Gene 33, 1984. The protein may be further purified by passage over a phosphocellulose column, as described by Blanco et al., 13 Nuc. Acid. Res. 1239, 1985. Blanco et al., id., also describe an exonuclease assay suitable for determination of inactivation of the exonuclease activity by genetic manipulation, as described below. Other enzymes associated with p2 and p3 in bacteriophage ϕ29 include p5 and p6, which increase the efficiency of polymerization by p2, as described by Salas, 109 Current Topics in Microbiology and Immunology 89, 1983.

Exonuclease Mutants

We shall now briefly describe the cloning of ϕ29 DNA polymerase and the manipulation of the p2 gene to produce examples of exonuclease mutants useful in this invention.

The starting plasmid was pBw2, which is a pBR322 derivative containing gene 2 of phage ϕ29, coding for the ϕ29 DNA polymerase, and including its ribosome-binding sequence (RBS) (Blanco et al. 29 Gene 33, 1984). In this construction the putative ATG initiation codon for the ϕ29 DNA polymerase is located 30 bp downstream a unique HindIII restriction site. Plasmid pBw2 was linearized with Hind III and subjected to a controlled digestion with the nuclease Bal3l. The DNA was then digested with the restriction nuclease ScaI, which cuts 444 base pairs downstream gene 2, and the 5' protruding ends were filled-in with the Klenow fragment of E. coli DNA polymerase I. The DNA fragment containing gene 2 was ligated with the T4 DNA ligase to plasmid pAZe3ss (Zaballos et al., 58 Gene 67, 1987) and digested with NcoI, whose 5' protruding ends were then filled-in using Klenow fragment. The ligation product was used to transform competent E. coli M72 cells (lysogenic for bacteriophage λ and containing the temperature-sensitive cI857 repressor) and ampicillin-resistant bacteria selected. The latter were replica-plated in plates containing ampicillin (100 μ/ml) by growing them overnight at 30°C, followed by 3 h at 42°C. The colonies were transfered to nitrocellulose filters and lysed with 0.1% sodium dodecyl sulfate. The filters were washed, incubated with rabbit anti-ϕ29 DNA polymerase serum (produced by standard procedure) and the ϕ29 DNA polymerase-containing colonies were detected by incubation with [^{125}I] protein A followed by autoradiography. DNA sequencing of the selected clones allowed selection of the recombinant plasmids pAZw200 and pAZa203, which include ϕ29 DNA starting at the ATG

triplets corresponding to position 2869-2867 and 2860-2858, respectively, in the open reading frame coding for p2, from the left ϕ29 DNA end (Yoshikawa et al., 17 Gene, 323, 1982). When the E. coli M72 cells, transformed with the recombinant plasmids pAZw200 or pAZa203, containing the gene coding for the ϕ29 DNA polymerase under the control of the P_L promoter of bacteriophage λ and with the RBS of gene ner of bacteriophage Mu, were grown at 30°C and then shifted to 42°C for 20 min to inactivate the λ CI857 repressor, followed by 2 h at 38°C, enzymatically active ϕ29 DNA polymerase was synthesized. About 150 and 300 μg of highly purified ϕ29 DNA polymerase was obtained per g of cells transformed with the recombinant plasmids pAZw200 and pAZa203, respectively.

The EcoRI-Hind III fragment from the recombinant plasmid pAZw200, containing the ϕ29 DNA polymerase gene and the RBS of gene ner of bacteriophage Mu was ligated, using T4 DNA ligase, to the EcoRI-HindIII sites of the replicative form of bacteriophage M13mp19. E. coli JM103 cells were transfected with such DNA and white plaques were selected in plates containing X-gal and isopropilthiogalactoside (IPTG). The selected plaques were amplified in liquid medium and the replicative form was isolated to check (by restriction analysis) the presence of the desired EcoRI-HindIII fragment. The single-stranded DNA was also isolated and used for site-directed mutagenesis, carried out as described by Nakamaya et al., 14 Nucl. Acids Res. 9679, 1986. The synthetic oligodeoxynucleotides used for the site-directed mutagenesis were:

1)

5' AGTTGTGCCTTTGAGAC

2)

5' GACTTTGCGACAACTAC

3)

5' CTCAAATTTGCCGGAGC

The recombinant clones containing point mutations were selected by hybridization to the corresponding mutagenic oligonucleotides 5' [^{32}P]-labeled with T4 polynucleotide Kinase and [Y-^{32}P] ATP. Single-stranded DNA was isolated from the selected clones and the sequence of the complete DNA polymerase gene was determined to check that each clone contained only the desired mutation. The EcoRI-BstBI fragment from the different clones was ligated with T4 DNA ligase to the same sites cf plasmid pABw2, which contains the EcoRI-HindIII fragment of plasmid pAZw200 cloned at the corresponding sites of plasmid pT7-3 of the pT7 series (Tabor et al. 82 Proc. Natl. Acad. Sci. USA, 1074, 1985), under the control of the ϕ10 promoter of bacteriophage T7. This EcoRI-BstBI fragment replaces the wild-type sequence in that region by the corresponding mutant sequence. In this way, the recombinant plasmids pABn2D12A, pABn2E14A, pABn2D66A, pABn2D12AD66A and pABn2E14AD66A were selected, containing the corresponding amino acid changes from the amino-terminal end of the ϕ29 DNA polymerase. The recombinant plasmids were used to transform E. coli BL21 (DE3) cells containing tne bacteriophage T7 RNA polymerase gene in the host DNA under the control of the lac uv5 promoter (Studier et al,, 189 J. Mol. Biol. 113, 1986) being, therefore, inducible by IPTG. The ampicillin-resistant bacteria were analyzed for the presence of recombinant plasmids. Expression of the ϕ29 DNA polymerase mutant proteins was obtained by addition of 1 mM IPTG to E. coli cells containing the recombinant plasmids, grown at 37°C and incubated for 1 h at 37°C. Five different mutant proteins were obtained, with the following amino acid changes: 1) alanine at position 12 (with reference to the first methionine in the gene encoding p2) in place of the natural aspartic acid (D12A); 2) alanine at position 14 instead of glutamic acid (E14A); 3) alanine at position 66 instead of aspartic acid (D66A); 4) alanine at positions 12 and 66 instead of aspartic acid (D12A, D66A); and 5) alanine at position 14 and 66 (E14A, D66A). The different mutant proteins were purified and their 3'-5' exonuclease activity determined by the above standard assay to be 100-1000 fold lower than that of the wild-type naturally occurring ϕ29 DNA polymerase.

Deposits

Strains pAZW200 (wild type p2 gene), pKC30Al (wild type p3 gene), pABN2D12AD66A (exonuclease deficient p2 gene having alanine at positions 12 and 66) were deposited under the Budapest Treaty on March 24, 1989, with the ATCC and assigned numbers 67920, 67918, 67919, respectively.

Referring to the Figure, the oligonucleotides used to form the above mutants were selected by taking into account the amino acid sequence homology with other polymerases and those mutations known to reduce exonuclease activity of DNA polymerase I. Derbyshire et al. 240 Science 199, 1988. Other mutations which are likely to produce suitable exonuclease mutants are shown in the black boxes. Generally, the amino acid at these portions is either deleted or replaced with a different amino acid. Large deletions or multiple replacement of amino acids are also useful in this invention. After mutagenesis, the level of exonuclease activity is measured and the amount of DNA polymerase activity determined to ensure it is sufficient for use in this invention (e.g., for DNA sequencing), being processive and having strand displacement activity.

Uses

Uses of DNA polymerases as hereinbefore described in accordance with the present invention include, for example, synthesis of long DNA probes containing multiple copies of a desired sequence, obtained by strand-displacement synthesis on single stranded DNA, including such long probes labelled with labelled dNTPs at a high specific activity; random labelling of double-stranded DNA at a high specific activity by using degenerated oligonucleotide primers; second-strand cDNA synthesis in cDNA cloning; random mutagenesis of single- and double-stranded DNA templates by using an exonuclease-deficient DNA polymerase under conditions of low DNA replication fidelity; site-directed mutagenesis on double stranded DNA templates and gene amplification or synthesis of long double-stranded DNA fragments using synthetic oligonucleotides as primers.

The modified ϕ29-type DNA polymerases herein described are particularly useful for performing a polymerase chain reaction to produce extremely long strands of DNA.

Example 1: P.C.R.

There follows an example of a polymerase chain reaction using ϕ29 DNA polymerase. In general, the DNA polymerase may simply be used in place of Klenow or Tag polymerases.

0.1 pmol of target DNA are mixed with 300 pmol each of selected oligonucleotides (15-20 mers), an 75 nmol of each deoxynucleoside triphosphate (1N5 mM) in 50 μl of a buffer containing 50 mM Tris-HCl (pH 7.5) and 10 mM magnesium chloride. The solution is brought to 95°C for 10 minutes, and cooled to 30°C for 1 min in a waterbath. 1 μl containing 20 ng of ϕ29 DNA polymerase (either wild type or an exonuclease mutant) is added to the mixture and the reaction allowed to proceed for 5 min at 30°C, after which the cycle of heating, cooling, adding enzyme, and reacting is repeated about nine times. The polymerase used is purified by standard procedures.

Prior polymerases used in polymerase chain reactions failed to provide DNA fragments in the size range greater than about 2 kilobases (Saiki et al., 239 Science 487, 1988; Keohavong et al., 71 Gene 211, 1988). This relative short size is probably due to the secondary structure and hinderance produced by reannealing of the DNA fragment, which impedes the progress of these DNA polymerases. Because ϕ29 DNA polymerase has a high processivity and strand displacement ability, it is an ideal enzyme for DNA amplification to produce long amplified molecules , particularly if modified to reduce exonuclease activity while retaining the aforementioned desirable characteristics.

Example 2: Synthesis of Long Strands of DNA

Modified ϕ29-type DNA polymerases as hereinbefore described permit ready synthesis of very long DNA molecules useful in a large number of applications, e.g. RFLP analysis, and DNA probe construction. There follows an example of this methodology employing a ϕ29 DNA polymerase.

Single-stranded M13 DNA was hybridized with a 17-mer M13 oligonucleotide primer. The incubation mixture contained, in 10 μl, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1mM DTT, 0.5 μg of primed M13 DNA, 80 μM each dCTP, dGTP, dTTP and [α-^{32}P] dATP and ϕ29 DNA polymerase (50 ng). After incubation for 40 min at 30°C the samples were filtered through Sephadex® G-50 spin columns in the presence of 0.1% sodium dodecyl sulfate and the Cerenkov radiation of the excluded fraction was counted. To analyze the size of the DNA synthesized, a sample was subjected to electrophoresis in alkaline 0.7% agarose gels along with DNA length markers. The DNA markers were detected with ethidium bromide and the synthesized DNA was detected by autoradiography of the dried gel. In 40 min of incubation at 30°C, DNA longer than 70 Kb was synthesized.

Claims

1. A method for amplification of a DNA sequence including the steps of annealing a first primer and a second primer to opposite strands of a double-stranded DNA sequence and incubating the annealed mixture with a DNA polymerase, wherein the DNA polymerase employed is a modified ϕ29-type DNA polymerase exhibiting less than 10% of the exonuclease activity of the corresponding naturally-occurring polymerase.

2. A method as claimed in claim 1 wherein said first and second primers have their 3' ends directed towards each other after annealing.

3. A method as claimed in claim 1 or claim 2 which further comprises, after said incubation step, denaturing the resulting DNA, annealing said first and second primers to the denatured DNA and incubating the last said annealed mixture with said polymerase.

4. A method as claimed in claim 3 wherein said cycle of denaturing, annealing, and incubating is repeated from 10-40 times.

5. A method as claimed in any one of claims 1 to 4 wherein said modified ϕ29-type DNA polymerase is derived from a DNA polymerase selected from ϕ29, Cp-1, PRD1, ϕ15, ϕ21, PZE, PZA, Nf, M2Y, B103, SF5, GA-1, Cp-5, Cp-7, PR4, PR5, PR722 and L17.

6. A method as claimed in any one of the preceding claims wherein said modified ϕ29-type DNA polymerase has no detectable exonuclease.

7. A method as claimed in any one of the preceding claims wherein the DNA sequence amplified is greater than 10 kilobases in length.

8. A method for production of DNA molecules of greater than 10 kilobases in length comprising:

providing a template DNA molecule;
annealing a primer with said template molecule;
and
incubating the annealed primer and template molecules in the presence of a modified ϕ29-type DNA polymerase and a mixture of four different deoxynucleoside triphosphates, said modified ϕ29-type DNA polymerase exhibiting less than 10% of the exonuclease activity of the corresponding naturally-occurring polymerase.

Patentansprüche

1. Verfahren zur Amplifizierung einer DNA-Sequenz, einschließend die Schritte des Anelierens bzw. Annealings eines ersten Primers und eines zweiten Primers zu gegenüberliegenden Strängen einer doppelsträngigen DNA-Sequenz und Inkubierens dieser anelierten Mischung mit einer DNA-Polymerase, wobei die angewandte DNA-Polymerase eine modifizierte DNA-Polymerase vom Typ ϕ29 ist, welche weniger als 10% der Exonucleaseaktivität der entsprechenden natürlich auftretenden Polymerase zeigt.

2. Verfahren nach Anspruch 1, bei dem die 3'-Enden des ersten und zweiten Primers nach der Anelierung zueinander gerichtet vorliegen.

3. Verfahren nach Anspruch 1 oder 2, welches nach diesem Inkubationsschritt ferner das Denaturieren der resultierenden DNA, das Anelieren des ersten und zweiten Primers an die denaturierte DNA und das Inkubieren dieser letzten anelierten Mischung mit der Polymerase umfaßt.

4. Verfahren nach Anspruch 3, bei dem der Zyklus aus Denaturieren, Anelieren und Inkubieren 10- bis 40mal wiederholt wird.

5. Verfahren nach einem der Ansprüche 1 bis 4, bei dem die modifizierte DNA-Polymerase vom Typ ϕ29 abgeleitet ist von einer aus ϕ29, Cp-1, PRD1, ϕ15, ϕ21, PZE, PZA, Nf, M2Y, B103, SF5, GA-1, Cp-5, Cp-7, PR4, PR5, PR722 und L17 gewählten DNA-Polymerase.

6. Verfahren nach einem der vorhergehenden Ansprüche, wobei die modifizierte DNA-Polymerase vom Typ ϕ29 keine nachweisbare Exonuclease aufweist.

7. Verfahren nach einem der vorhergehenden Ansprüche, wobei die amplifizierte DNA-Sequenz mehr als 10 Kilobasen lang ist.

8. Verfahren zur Herstellung von DNA-Molekülen mit einer Länge von mehr als 10 Kilobasen, umfassend:

Vorsehen eines Template-DNA-Moleküls;
Anelieren eines Primers mit dem Template-Molekül;
und
Inkubieren des anelierten Primers und der Template-Moleküle in Gegenwart einer modifizierten DNA-Polymerase vom Typ ϕ29 und einer Mischung von vier unterschiedlichen Desoxynucleosidtriphosphaten, wobei die modifizierte DNA-Polymerase vom Typ ϕ29 weniger als 10% Exonucleaseaktivität der entsprechenden natürlich auftretenden Polymerase zeigt.

Revendications

1. Méthode pour amplifier une séquence d'ADN comprenant les étapes consistant à hybrider une première amorce et une seconde amorce avec les brins opposés d'une séquence d'ADN double brin et à incuber le mélange hybridé avec une ADN polymérase, où l'ADN polymérase utilisée est une ADN polymérase de type ϕ29 modifiée, présentant moins de 10 % de l'activité exonucléasique de la polymérase naturelle correspondante.

2. Méthode telle que revendiquée à la revendication 1 dans laquelle lesdites première et deuxième amorces ont leurs extrémités 3' dirigées l'une vers l'autre après hybridation.

3. Méthode telle que revendiquée à la revendication 1 ou 2, qui comprend en outre, après ladite étape d'incubation, les étapes consistant à dénaturer l'ADN résultant, hybrider lesdites première et deuxième amorces avec l'ADN dénaturé et incuber ledit dernier mélange hybridé avec ladite polymérase.

4. Méthode telle que revendiquée à la revendication 3 dans laquelle ledit cycle de dénaturation, hybridation, et incubation est répété de 10-40 fois.

5. Méthode telle que revendiquée dans l'une quelcon-

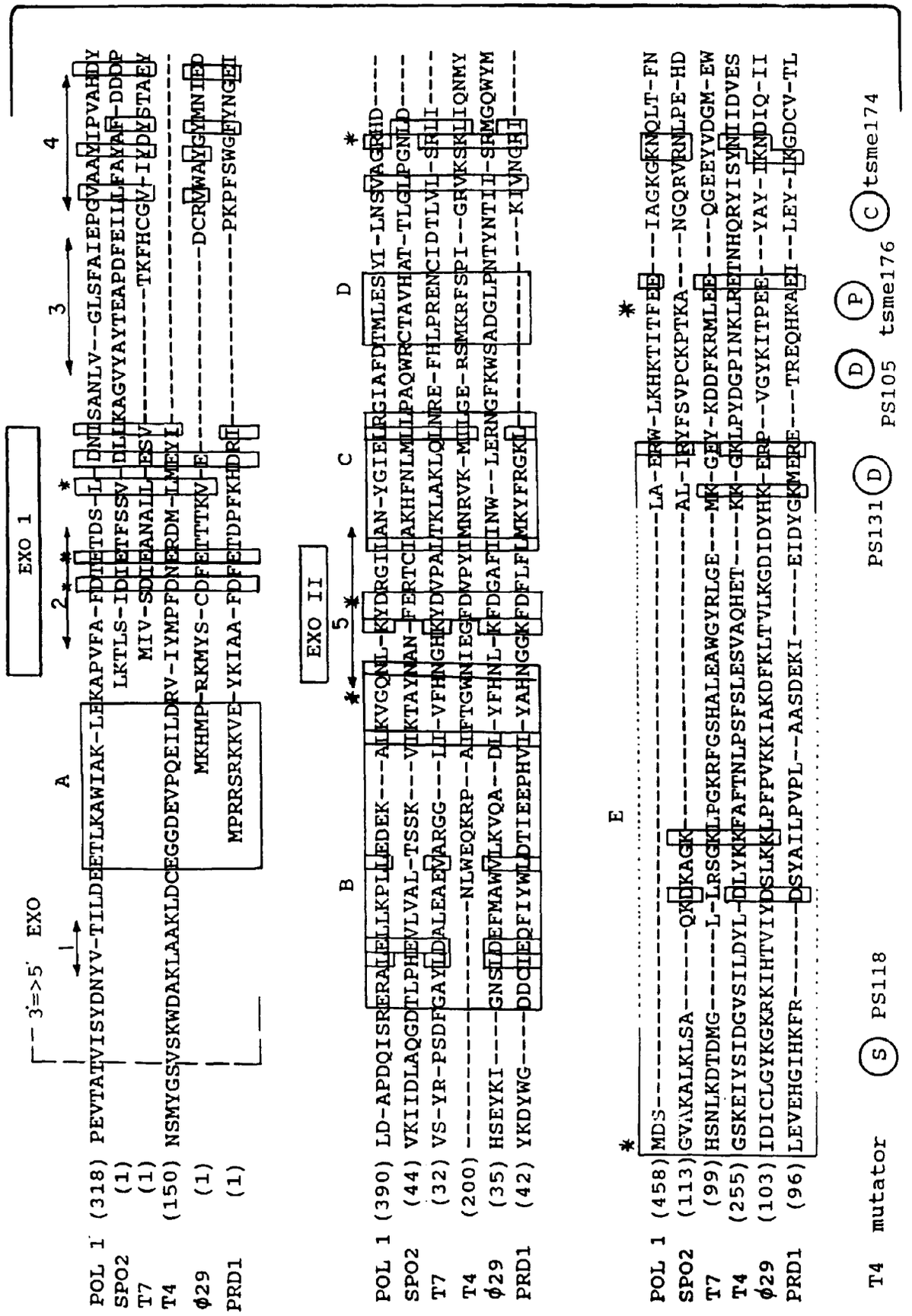
que des revendications 1 à 4, dans laquelle ladite ADN polymérase de type ϕ29 modifiée est dérivée d'une ADN polymérase choisie parmi ϕ29, Cp-1, PRD1, ϕ15, ϕ21, PZE, PZA, Nf, M2Y, B103, SF5, GA-1, Cp-5, Cp-7, PR4, PR5, PR722 et L17.

6. Méthode telle que revendiquée dans l'une quelconque des revendications précédentes dans laquelle ladite ADN polymérase de type ϕ29 modifiée n'a pas d'exonucléase détectable.

7. Méthode telle que revendiquée dans l'une quelconque des revendications précédentes dans laquelle la séquence d'ADN amplifiée a une longueur supérieure à 10 kilobases.

8. Méthode de production de molécules d'ADN d'une longueur supérieure à 10 kilobases comprenant les étapes consistant à :

fournir une molécule d'ADN matrice ;
hybrider une amorce avec ladite molécule matrice ; et
incuber l'amorce hybridée et les molécules matrices en présence d'une ADN polymérase de type ϕ29 modifiée et un mélange de quatre désoxynucléosides triphosphates différents, ladite ADN polymérase de type ϕ29 modifiée présentant l'activité exonucléasique moins de 10% de la polymérase naturelle correspondante.


EXO 1
EXO II
3'=>5' EXO
A
B
C
D
E
1
2
3
4
5
POL 1 (318) PEVTATVISYDNYV-TILDEETLKAWIAK-LEKAPVFA-FDTETDS-L-DNDSANLV--GLSFAIEPGVAAMLPVAHDY
SPO2 (1) LKTLS-IDIETFSSV-DLIKAGVVAYTEAPDFEIILFAVAF-DDDP
T7 (1) MIV-SDIEANAIL-ESV-TKFHCGV-IYDVSTAEY
T4 (150) NSMYGSVSKWDAKLAAKLDCEGGDEVPQEILDRV-IYMPFDNERDM-LMEYI-DCRVWAVGYMNIED
ϕ29 (1) MKHMP-RKMYS-CDFETTKV-E-PKPFSWGFYNGEI
PRD1 (1) MPRRSRKKVE-YKIAA-FDFETDPFKHDRI-
POL 1 (390) LD-APDQISRERALELLKPLLEDEK---ALKVGQML-KYDRGIIAN-YGIELRGIAFDTMLESYI-LNSVAGRHD----
SPO2 (44) VKIIDLAQGDTLPHEVLVAL-TSSK---VIKTAYNAN-FERTCIAKHFNLMLLPAQWRCTAVHAT-TLGLPGNLD----
T7 (32) VS-YR-PSDFGAYLDALEAEVARGG---LI-VFHNGHKYDVPALTKLAKLQLNRE-FHLPRENCIDTLVL-SRLI----
T4 (200) ----------NLWEQKRP--AIFTGWNIEGFDVPYIMNRVK-MLLGE-RSMKRFSPI--GRVKSKLIQNMY
ϕ29 (35) HSEYKI----GNSIDEFMAWVLKVQA--DL-YFHNL-KFDGAFIINW--LERNGFKWSADGLPNTYNTII-SRMGQWYM
PRD1 (42) YKDYWG----DDCIEQFIYWLDTIEEPHVI-YAHNGGKFDFLFLMKYFRGKL----KIVNGHI-----
POL 1 (458) MDS----------LA-ERW-LKHKTITFEE--IAGKGKNQLT-FN
SPO2 (113) GVAKALKLSA-----QKDKAGK----------AL-IRYFSVPCKPTKA----NGQRVRNLPE-HD
T7 (99) HSNLKDTDMG-----L-LRSGKLPGKRFGSHALEAWGYRLGE---MK-GEY-KDDFKRMLEE---QGEEYVDGM-EW
T4 (255) GSKEIYSIDGVSILDYL-DLYKKFAFTNLPSFSLESVAQHET----KK-GKLPYDGPINKLRETNHQRYISYNIIDVES
ϕ29 (103) IDICLGYKGKRKIHTVIYDSLKKLPFPVKKIAKDFKLTVLKGDIDYHK-ERP--VGYKITPEE--YAY-IKNDIQ-II
PRD1 (96) LEVEHGIHKFR-----DSYAILPVPL--AASDEKI-----EIDYGKKMERE--TREQHKAEI--LEY-LKGDCV-TL
T4 mutator (S) PS118 PS131 (D) PS105 (D) tsme176 (P) (C) tsme174

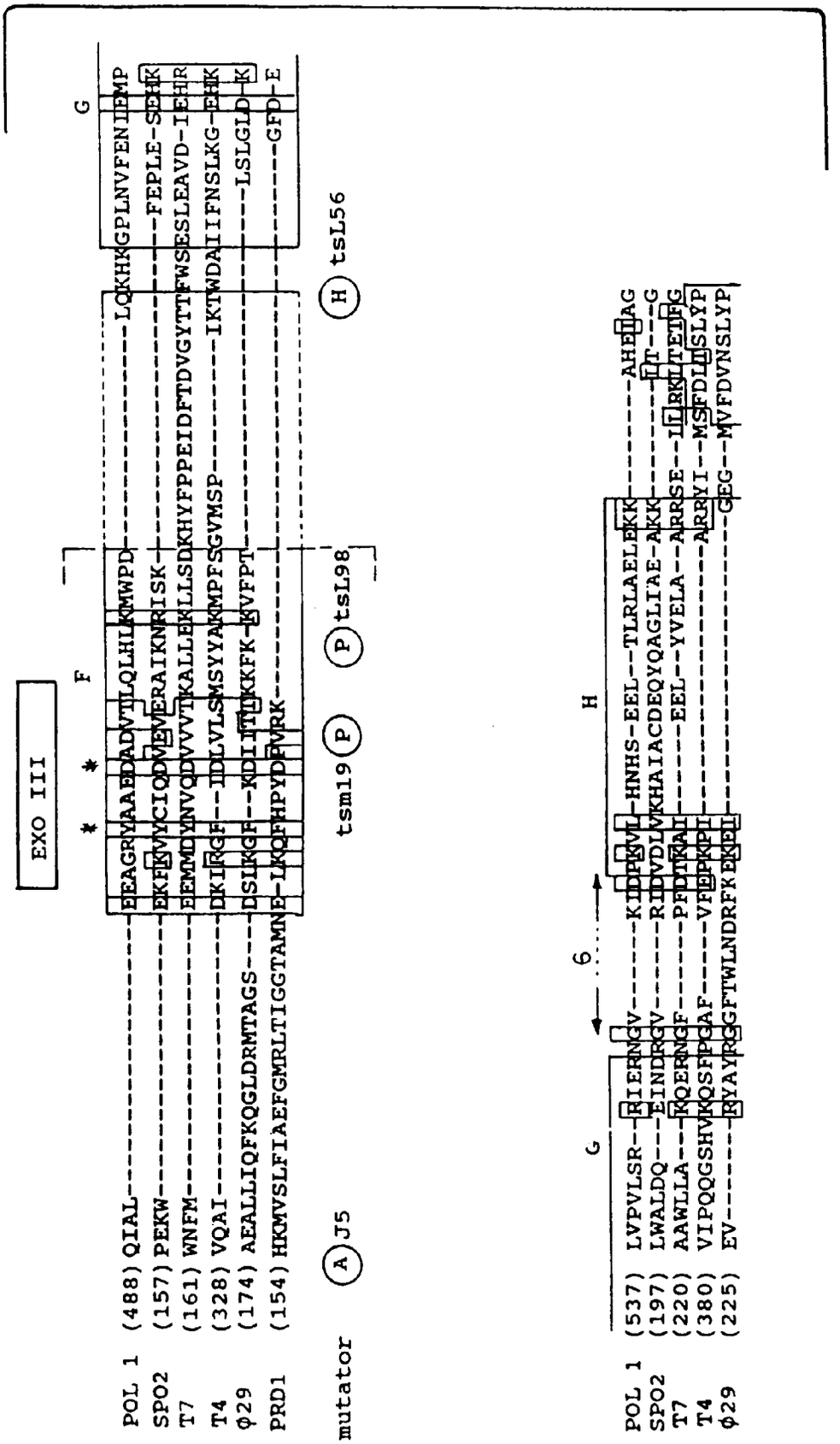

EXO III
F
G
H
POL 1 (488) QIAL
SPO2 (157) PEKW
T7 (161) WNFM
T4 (328) VQAI
φ29 (174) AEALLIQFKQGLDRMTAGS
PRD1 (154) HKMVSLFIAEFGMRLTIGGTAMN
T4 mutator
A J5
tsm19 P
P tsL98
H tsL56
6
POL 1 (537) LVPVLSR
SPO2 (197) LWALDQ
T7 (220) AAWLLA
T4 (380) VIPQQGSHVKQSFPGAF
φ29 (225) EV

FIG. CONT